Sarcomere length influences μ-calpain-mediated proteolysis of bovine myofibrils

A. D. Weaver, B. C. Bowker and D. E. Gerrard

J Anim Sci 2009.87:2096-2103.
doi: 10.2527/jas.2008-1317 originally published online Mar 27, 2009;

Sarcomere length influences μ-calpain-mediated proteolysis of bovine myofibrils

A. D. Weaver,*[1] B. C. Bowker,† and D. E. Gerrard*[2]

*Department of Animal Sciences, Purdue University, West Lafayette, IN 47907; and †USDA-ARS, Food Quality Laboratory, Beltsville, MD 20705

ABSTRACT: Muscle shortening and postmortem proteolysis both influence beef tenderness, but their interacting effects on tenderness are relatively unknown. Inherent myofibril structure and the extent of overlap between myosin and actin filaments are hypothesized to affect the availability of substrates for degradation by calpains. The objective of this study was to determine the influence of sarcomere length on the extent of calpain-induced proteolysis of bovine myofibrils in vitro. Bovine semitendinosus muscles were excised within 20 min postmortem and dissected into strips, which were stretched and attached to applicator sticks or allowed slack to generate samples with different sarcomere lengths upon rigor completion. Samples were allowed to undergo rigor in a neutral pH buffer containing a protease inhibitor. Myofibrils were isolated and incubated at room temperature with excess exogenous μ-calpain at a ratio of 1:800 (wt/wt; enzyme:myofibrillar protein) at pH 6.8 for 0, 2, 60, 1,440, or 2,880 min. Purified troponin was subjected to the same digestion conditions. Proteolysis of troponin T (TnT) was monitored using SDS-PAGE and Western blotting. Sarcomere length was greater ($P < 0.0001$) in stretched versus shortened samples (2.99 μm ± 0.03 vs. 2.12 ± 0.03 μm, respectively, means ± SE). Western blots for both stretched and shortened samples exhibited bands corresponding to intact TnT and TnT fragments. The abundance of intact TnT decreased ($P < 0.0001$) with incubation time across both treatments. At 1,440 and 2,880 min, less ($P < 0.05$) intact TnT was detected in samples with long sarcomeres. These data indicate proteolysis of TnT occurs to a greater extent in samples with longer sarcomeres, possibly due to easier access of proteases to their targeted substrates. Degradation patterns of TnT were qualitatively similar between myofibrils and purified troponin after incubation with μ-calpain. Therefore, it is unlikely that the mechanism by which proteolysis is limited in short sarcomeres involves an actomyosin-mediated interference of TnT.

Key words: beef, μ-calpain, sarcomere length, tenderness, troponin-T

J. Anim. Sci. 2009. 87:2096–2103
doi:10.2527/jas.2008-1317

INTRODUCTION

Both sarcomere shortening and postmortem proteolysis have a direct effect on meat tenderness. Wheeler and Koohmaraie (1994) reported that sarcomere shortening is responsible for tenderness values early postmortem (<24 h), whereas variations in proteolysis via the calpain system are responsible for differences in tenderness during aging. Although both factors independently affect meat tenderness, the specific influence of sarcomere length (muscle shortening) on proteolysis remains unclear. Evidence for a potential interaction between sarcomere shortening and proteolysis is provided by numerous investigators reporting that cold-shortened beef is resistant to aging (Davey et al., 1967; Davey and Gilbert, 1976; Locker, 1982). Locker (1982) suggested that tenderness of cold-shortened meat may be compromised by the increased overlap of thick and thin filaments, which inhibits protease access to intrasarcomeric substrates. Conversely, several studies compared cold-shortened to normal LM and concluded that sarcomere length does not affect the extent of proteolysis (Jaime et al., 1992; Wheeler and Koohmaraie, 1999). Using an in vivo model with a wider range of sarcomere lengths than had been tested in previous studies, Weaver et al. (2008) determined that sarcomere length contributes to differences in the extent of postmortem proteolysis of troponin T (**TnT**) in excised beef semitendinosus muscle. Weaver et al. (2008) hypothesized that sarcomere length-related differences in proteolysis may be the result of differences in substrate availability. To more precisely test the hypothesis that sarcomere length in-

[1]Present address: Department of Animal and Range Sciences, South Dakota State University, Brookings 57007.
[2]Corresponding author: dgerrard@vt.edu
Received July 22, 2008.
Accepted February 5, 2009.

fluences TnT proteolysis in myofibrils, an in vitro model system was utilized in the current study in which the extent of TnT proteolysis was quantified in myofibrils with divergent sarcomere lengths after incubation with an excess of exogenous μ-calpain. Furthermore, little is known concerning the mechanism by which sarcomeric structures, such as actomyosin bonds, influence access of proteolytic enzymes to their substrates. Thus, a secondary objective of this study was to determine the influence of rigor bonds on μ-calpain-mediated proteolysis of troponin by comparing the degradation patterns of myofibrils and purified troponin after in vitro incubation with excess μ-calpain.

MATERIALS AND METHODS

Two A-maturity market steers were harvested at the Purdue University Meat Science, Teaching and Research Laboratory under standard harvesting procedures.

Muscle Samples

The semitendinosus was removed from 1 side of each carcass approximately 20 min postmortem. External fat and connective tissue were removed. The superficial portion of the semitendinosus was dissected parallel to muscle fiber orientation into strips measuring ~0.5 cm wide and ~10 cm in length. Two methods were utilized to generate a range of sarcomere lengths: prerigor muscle strips were either: 1) stretched between 110 and 160% of original length and restrained on wooden applicator sticks to generate long sarcomeres or 2) allowed varied amounts of slack to generate short sarcomeres. After attachment to wooden applicator sticks, cut or frayed fibers were removed from the outer surface of each strip to ensure development of uniform sarcomere lengths. Samples were immersed in rigor buffer (**RB**; sarcomere length) [75 m*M* KCl, 10 m*M* imidazole; pH 7.2, 2 m*M* $MgCl_2$, 2 m*M* EGTA, and 1 m*M* NaN_3, with 0.1 m*M* phenylmethylsulfonyl fluoride (**PMSF**) to inhibit endogenous proteolytic activity] and held at 4°C overnight with stirring. Twenty-four hours postmortem, samples were removed from applicator sticks, ends and additional frayed fibers were removed from the samples, and myofibrils were prepared for sarcomere length determination and in vitro digestion assays.

Myofibril Preparation

Muscle strips were minced with a scalpel and 1-g samples were homogenized in 5 mL of RB + PMSF for two 10-s bursts using a Polytron homogenizer (Brinkmann Instruments, New York, NY) at medium speed (speed setting 6). An additional 5 mL of RB + PMSF was added to the suspension. Samples were centrifuged at 1,000 × *g* for 10 min at 4°C. The pellet was washed and resuspended 2 more times. The final myofibril pellet was resuspended with 5 mL of RB + PMSF and diluted to 50% with glycerol for storage at −20°C.

Sarcomere Length Determination

Myofibrils were prepared for direct measurement of sarcomere length using phase contrast microscopy as described by Weaver et al. (2008). The average of 5 sarcomeres was determined across 20 myofibrils per sample. To fulfill our objective of studying proteolysis between samples with divergent sarcomere lengths, samples representing each treatment (stretched or shortened) were selected from each animal based on quality of preparation and uniformity of sarcomere length. Samples with increased abundance of fragments (<5 sarcomeres per myofibril) or large bundles of myofibrils were excluded. After sarcomere length determination on all samples generated, specific samples, with different sarcomere lengths, were selected for digestion with μ-calpain,

μ-Calpain Incubation of Myofibrils and Purified Troponin

Isolated myofibrils of selected samples were washed and digested according to Huff-Lonergan et al. (1996) with modifications. Purified troponin from bovine semitendinosus (kindly provided by Darl Swartz, Purdue University) was also subjected to μ-calpain-mediated digestion. The purified troponin protein contained all 3 subunits of the protein (TnT, troponin I, and troponin C). The purity of troponin was validated by SDS-PAGE. Estimated molecular weights (**MW**) for TnT, troponin I, and troponin C were 40.8, 23.6, and 20.3 kDa, respectively (data not shown). Protein concentration was determined using the Biuret method and samples were adjusted with reaction buffer (165 m*M* KCl, 50 m*M* imidazole, pH 6.8) to 4 mg of protein/mL, 100 μ*M* $CaCl_2$, and 15 m*M* β-mercaptoethanol. The digestion of the myofibril and troponin samples was initiated by the addition of μ-calpain (calpain-1, porcine erythrocyte, EC 3.4.22.17, Calbiochem, LaJolla, CA, specific activity 120 units/mg of protein) in reaction buffer at a ratio of 1:800 (wt/wt; enzyme:myofibrillar protein). One unit is defined as the amount of enzyme that will increase the absorbance at 750 nm by 1.0 in 30 min at 30°C. Using SDS-PAGE, the purity (>90%) and composition of the μ-calpain was found to be similar to that of Lametsch et al. (2004). Samples were incubated at room temperature and aliquots were removed from each sample at 0, 2, 120, 1,440, and 2,880 min postincubation. Control samples (0 min) were removed before addition of protease. To account for residual endogenous protease activity, an aliquot of each sample was incubated at room temperature for 2,880 min in reaction buffer without the enzyme. One myofibril sample was digested with μ-calpain in the presence of 20 m*M* EDTA to inhibit Ca^{++}-dependent proteolysis. To stop the reaction, aliquots were removed and added to a 0.5 volume of sample buffer [3 m*M* EDTA, 3% wt/vol SDS, 20% vol/vol glycerin, 0.003% wt/vol Pyronin Y (Sigma-Aldrich, St. Louis, MO), 30m*M* Tris-HCl, pH 8.0] and a 0.1 volume of β-mercaptoethanol resulting in

a final protein concentration of ~2 mg/mL. Troponin samples were further diluted to 0.2 mg/mL in sample buffer. All samples were immediately denatured in a water bath at 100°C for 5 min, cooled, and then stored at −20°C until further analysis.

Gel Electrophoresis and Western Blotting

Denatured myofibril (10 μg) and purified troponin (1 μg) samples were loaded on 15% SDS-polyacrylamide resolving gels with an acrylamide:bisacrylamide weight ratio of 37.5:1 (Ready Gel, Tris-HCl gels, Bio-Rad Laboratories, Hercules, CA). A broad range (200 kDa to 6.5 kDa) of biotinylated MW standard (1.2 μg/lane; Bio-Rad Laboratories) was also loaded on each gel. Gels were run on the Bio-Rad Criterion Cell system (Bio-Rad Laboratories) at constant 200 V for 80 min at 4°C. Upper and lower chamber running buffer consisted of 25 m*M* Tris, 192 m*M* glycine, 2 m*M* EDTA, and 0.1% wt/vol SDS. Gels were equilibrated for 30 min at room temperature in transfer buffer (25 m*M* Tris, 192 m*M* glycine, and 15% vol/vol methanol) after electrophoresis and transferred to polyvinylidene difluoride membranes at 4°C using a Criterion blotter (Bio-Rad Laboratories) at a constant 90 V for 45 min. Blots were blocked overnight in blocking solution [PBS containing 1% vol/vol Tween 20 (**PBST**)] and 5% nonfat dry milk at 4°C with agitation. The following Western blotting procedures were completed at room temperature. Blots were triple-washed in PBST for 10 min per wash and incubated with a TnT monoclonal antibody (JLT-12, Sigma-Aldrich, St. Louis, MO) diluted 1:30,000 in PBST containing 0.1% wt/vol BSA (**PBST-BSA**) for 90 min. After incubation with primary antibody, blots were triple-washed in PBST and then incubated for 60 min with a goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories) diluted to 1:25,000 in PBST-BSA. Blots were again washed 3 times in PBST and bands were detected using an Amplified Opti-4CN substrate kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Immunoreactive bands were identified and images were captured. Blots were then incubated in stripping buffer (100 m*M* β-mercaptoethanol, 2% SDS wt/vol, 62.5 m*M* Tris-HCl, pH 6.8) for 30 min at 55°C. After stripping, blots were washed 3 times in PBST, 10 min per wash, and blocked overnight at 4°C in PBST with 5% nonfat dry milk. Blots were then relabeled with a monoclonal antiactin (5C5, Sigma-Aldrich) diluted 1:10,000 in PBST-BSA. Blots were washed, labeled with secondary antibody, and bands were detected as described above. Six blots were analyzed per treatment. To account for potential blot-to-blot variations in transfer efficiency, the same MW standards were run on each blot as a reference lane. The TnT and actin bands were identified and quantified using Kodak 1D software, version 2.0 (Eastman Kodak, Rochester, NY). Bands of TnT with MW of 41.7, 40.9, and 39.9 kDa were considered to be intact TnT and the sum of their intensities was quantified. To account for slight lane-to-lane variations in protein load, intact TnT bands within a lane were quantified relative to the actin band intensity within the same lane. The ratio of intact TnT intensity to actin intensity was then normalized to control (0 min) samples. Bands of TnT with MW of 38.0, 36.8, 33.9, 32.5, and 30.2 kDa were considered to be degradation products of TnT and were not included in the quantitative analysis.

Statistical Analysis

The fixed effects of digestion time (0, 2, 120, 1,440, and 2,880 min) and treatment (stretch vs. shortening) and the random effect of animal on TnT degradation were evaluated using the PROC MIXED procedure (SAS Institute Inc., Cary, NC) with a repeated measures design. Treatment means were separated using a least squares difference test.

RESULTS AND DISCUSSION

Myofibril samples with large differences in sarcomere length were generated in this study using protocols that controlled temperature declines and endogenous protease activity. No differences ($P = 0.20$) in sarcomere length were observed between animals. Sarcomere length was greater ($P < 0.0001$) in stretched samples compared with those allowed slack to shorten (2.99 ± 0.03 μm vs. 2.12 ± 0.03 μm, respectively, means ± SE). Micrographs of representative samples showing the different degrees of thick and thin filament overlap are shown in Figure 1. The approximate length of the thick filament is 1.5 μm (Page and Huxley, 1963; Bendall and Voyle, 1967) and the thin filament is 1.3 μm (Ringkob et al., 2004) in each bovine half-sarcomere; therefore, a sarcomere length of 2.12 μm allows for maximal rigor bond formation as the thin filament overlaps the entire span of the thick filament. In longer sarcomeres (2.99 μm), the overlap region is only ~0.2 to 0.6 μm in each half-sarcomere, resulting in substantially fewer rigor bonds (Huxley, 1967).

Western blotting revealed similar TnT banding patterns between samples with long and short sarcomeres after digestion with μ-calpain (Figure 2). This degradation pattern was also similar to excised semitendinosus samples aged up to 10 d postmortem (Weaver et al., 2008). This result is consistent with other reports showing that, in vitro, μ-calpain has the ability to degrade myofibrillar proteins in a manner similar to that observed with in vivo conditioning (Koohmaraie, 1988; Huff-Lonergan et al., 1996). Five bands were detected in 0 min (control) samples corresponding to MW of 41.7, 40.9, 39.9, 38.0, and 36.8 kDa. The first 3 bands, which are likely intact TnT isoforms, are consistent with those reported previously in whole muscle protein extracts of semitendinosus (Weaver et al., 2008). Muroya et al. (2006) also reported the presence of 3 bovine

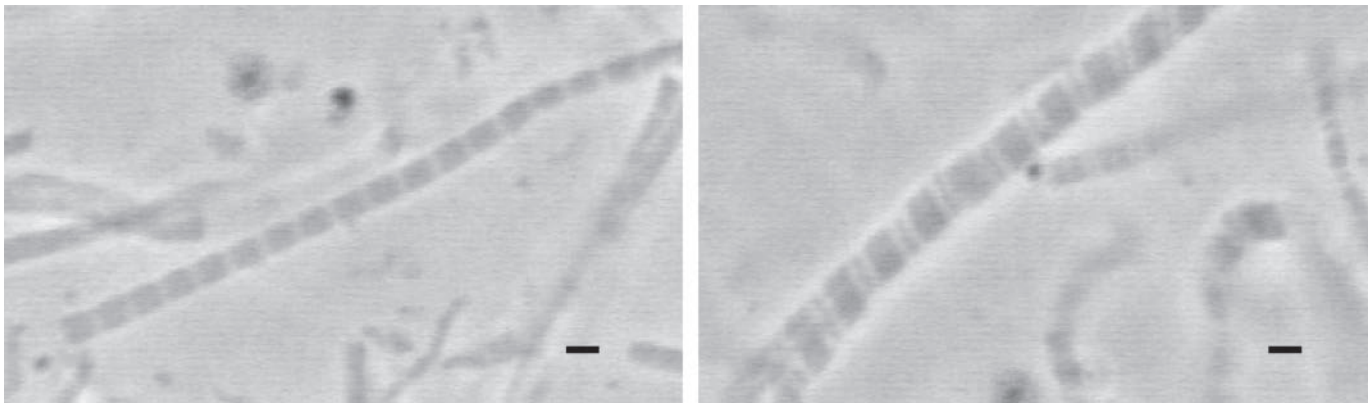

Figure 1. Representative micrographs of myofibrils with short (2.12 μm ± 0.03, left) and long sarcomeres (2.99 μm ± 0.03, right). Magnification (1,000×). Bar = 2 μm.

fast TnT isoforms in beef LM. Estimated MW of intact TnT isoforms are in agreement with others reporting MW of ~40 kDa for the intact protein (Negishi et al., 1996; Hughes et al., 2001). Little change was observed in the 38.0-kDa band over time in either sample and it is not clear if this band represents a degradation product or a fourth intact TnT isoform. Therefore, this band was not included during quantification of intact TnT. After incubation with μ-calpain for 60 min, both stretched and shortened samples yielded 3 additional bands with MW of 33.9, 32.5, and 30.2 kDa that appeared to increase in intensity over time. The 33.9-kDa fragment appeared as a minor band and was more apparent in short samples. Ertbjerg et al. (1999) also detected TnT degradation products corresponding to MW of 32 and 30 kDa. Due to the nature of proteolytic degradation and the specificity of the TnT antibody, it is possible that some TnT fragments may be further degraded or that some fragments may not even be detected using immunoblotting. As a result, it is difficult to definitively account for all of the degradation products of TnT. Therefore, only the disappearance of the intact TnT bands was quantitatively analyzed and the appearance of bands with MW of 38.0, 36.8, 33.9, 32.5, and 30.2 was not quantified.

Interestingly, the greatest MW intact TnT band (MW 41.7 kDa) appeared to degrade more rapidly than the 2 smaller intact bands (MW 40.9 and 39.9 kDa, respectively) in both treatments. The 41.7-kDa band remains evident after incubation with μ-calpain for 2,880 min in samples with short sarcomeres; however, only traces are apparent in long samples, suggesting that this isoform may be more susceptible to degradation by μ-calpain in long sarcomeres. A multitude of TnT isoforms exist that vary in size, AA sequence, and ultimately protein structure (Perry, 1998). Because calpains primarily target substrates based on structure (Tompa et al., 2004), it is possible that slight differences in the quaternary structure of this isoform allow for easier access to calpain cleavage sites, resulting in increased degradation compared with the other 2 isoforms; however, further studies are necessary to confirm this hypothesis.

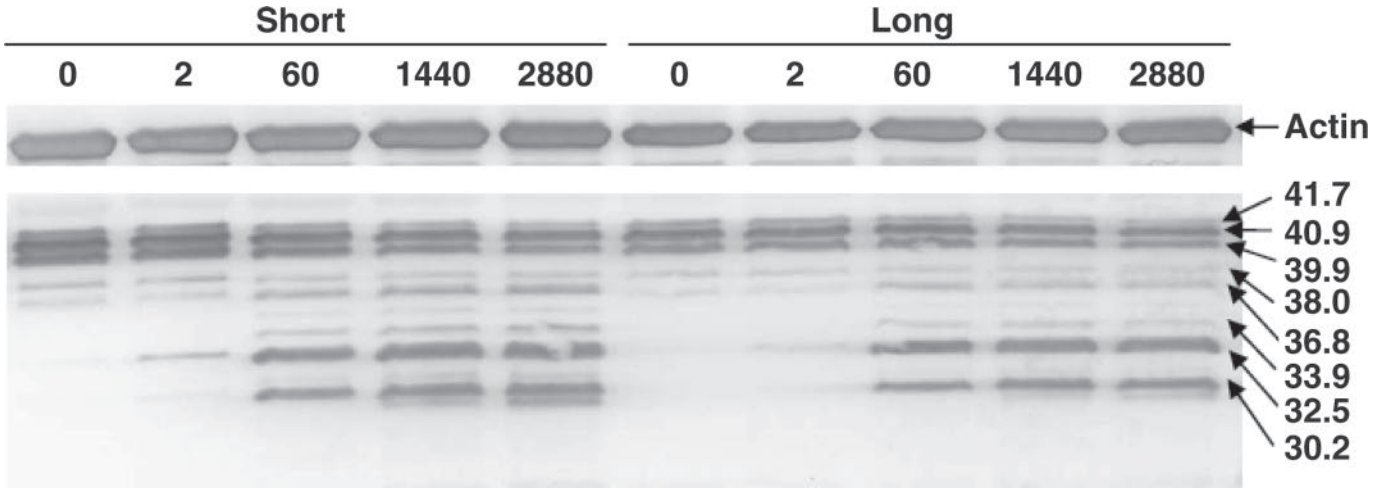

Figure 2. Western blot, prepared from a 15% resolving gel transferred to a polyvinylidene difluoride membrane, of bovine semitendinosus myofibrils digested with μ-calpain at a ratio of 1:800 (wt/wt; enzyme:myofibrillar protein) for 0, 2, 60, 1,440, or 2,880 min at room temperature in the presence of 100 μ*M* $CaCl_2$ and 15 m*M* β-mercaptoethanol from samples with short and long sarcomeres. Blot labeled with monoclonal troponin T antibody (clone JLT-12, Sigma-Aldrich, St. Louis, MO) and relabeled with monoclonal actin (clone 5C5, Sigma-Aldrich). Arrows indicate molecular weights of immunoreactive bands.

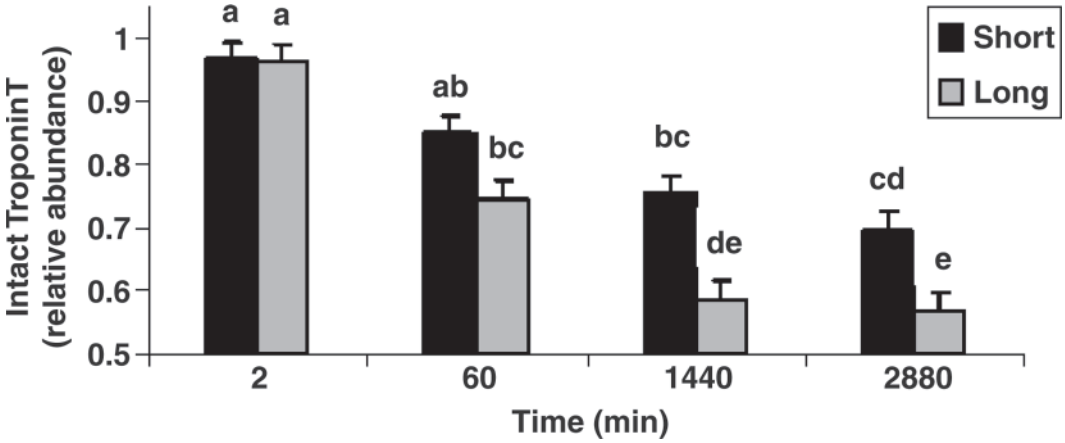

Figure 3. Disappearance of intact troponin T (TnT; 41.7-, 40.9-, and 39.9-kDa bands representing TnT isoforms) in bovine myofibrils isolated from bovine semitendinosus and digested with μ-calpain for 2, 60, 1,440, or 2,880 min. Abundance of intact TnT for all incubation times is expressed relative to actin and the abundance of TnT at 0 min. Bars represent mean ± SE. [a–e]Bars bearing different letters differ ($P < 0.05$).

Differences in the degradation of intact TnT between samples with long and short sarcomeres were quantified. The overall intensity of intact TnT was calculated as the sum of the intensity of the bands representing the 3 TnT isoforms (41.7-, 40.9-, and 39.9-kDa bands). The relative intensity of intact TnT decreased ($P < 0.0001$) with incubation time (Figure 3) in both treatments. No differences were observed in the abundance of intact TnT between long and short samples at 2 or 60 min; however, at 1,440 and 2,880 min, less ($P < 0.05$) intact TnT was detected in samples with long sarcomeres. These data indicate that proteolysis of TnT occurs to a greater extent in samples with longer sarcomeres, possibly due to easier access of proteases to their targeted substrates. The fact that no significant differences in TnT degradation were observed until 1,440 min of incubation with μ-calpain suggests that there may be a population of TnT molecules that is easily accessible in both treatments and after this population is initially degraded, access to remaining substrate is limited.

There are several possible explanations for differences in substrate accessibility between long and short sarcomeres. It is important to note that samples with long and short sarcomeres produced identical degradation products and the same general degradation pattern. These data suggest that potential calpain cleavage sites are not completely blocked in short samples but rather these sites may be hindered in short sarcomeres due to steric hindrance from other proteins or the presence of an actomyosin bond, or both. Calpains localize at the Z-lines (Goll et al., 2003) and likely must diffuse some distance to access potential substrates throughout the entire span of the sarcomere. In short sarcomeres, the thick filament and associated rigor bonds would be in close proximity if not in direct contact with the Z-line. Thus, the dense matrix created by intact rigor bonds could inhibit the migration of calpains to substrates, in turn slowing postmortem proteolysis but not inhibiting the process. In the case of myofibrils with longer sarcomeres, a greater portion of the thin filaments located close to the Z-line would be free of interactions with the thick filament, thus allowing easier access to substrates in this region, such as TnT. Likewise, it is possible that other proteins such as titin and nebulin hinder substrate binding sites in shorter sarcomeres. These extremely large structural proteins must change their conformation when sarcomeres shorten. This increased bunching of large proteins into small compartments could potentially limit access to cleavage sites on TnT.

To investigate further the hypothesis that thick and thin filament overlap influences the ability of calpains to access binding sites on TnT via the presence of rigor bonds, purified troponin was digested with μ-calpain. In comparing the in vitro digestion of myofibril versus purified troponin, it must be noted that differences in enzyme:substrate ratios between these 2 model systems may have influenced the data. Thus, only qualitative differences between banding patterns could be assessed between the myofibril and purified troponin digestions. The same 5 bands were detected at 0 min in purified troponin as those detected in myofibrils incubated with μ-calpain (Figure 4). Three degradation products with MW of 33.9, 32.5, and 30.2 kDa were evident by 2 min, whereas these bands were not detected in myofibril samples until 60 min. This result, not surprisingly, suggests calpains accessed cleavage sites more readily in purified troponin than those located in intact myofibrils. The intensity of intact TnT isoforms (MW 41.7, 40.9, 39.9) decreased over time, consistent with myofibril digestion. The intensity of the immunoreactive 38.0-kDa band decreased with incubation time, whereas little change was evident in the 36.8-kDa band. This result is in slight contrast to myofibril digestion in which the 38.0-kDa band shows little change over time, whereas the 36.8-kDa band increases slightly with time. The 33.9-, 32.5-, and 30.2-kDa degradation products increased in intensity corresponding to the decrease in intact TnT. Given that the degradation pattern of isolated TnT was similar to myofibrils incubated with μ-calpain, it is unlikely

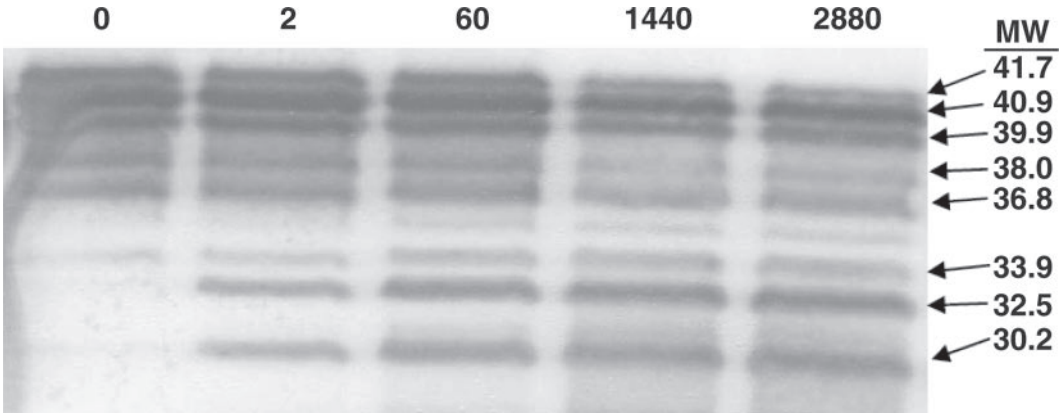

Figure 4. Western blot, prepared from a 15% resolving gel transferred to a polyvinylidene difluoride membrane, of purified troponin from bovine semitendinosus digested with μ-calpain at a ratio of 1:800 (wt/wt; enzyme:protein) for 0, 2, 60, 1,440, or 2,880 min at room temperature in the presence of 100 μ*M* $CaCl_2$ and 15 m*M* β-mercaptoethanol. Blot is labeled with a monoclonal troponin antibody (clone JLT-12, Sigma-Aldrich, St. Louis, MO). Arrows indicate molecular weights (MW) of immunoreactive bands.

that the mechanism by which proteolysis is limited in short sarcomeres involves an actomyosin-mediated interference of TnT. However, the role actomyosin bonds may play in slowing access to susceptible sites on TnT remains to be determined.

In these experiments, it was important to account for TnT proteolysis that occurred during the process of protein isolation and TnT proteolysis that may have occurred during the incubation period due to residual proteases. Figure 2 shows that time 0 myofibril samples contained only intact TnT and very little, if any, TnT degradation products. Such data indicate that minimal TnT proteolysis occurred during the process of isolating the myofibrils. Similarly, little TnT degradation was observed in purified troponin samples at time 0 (Figure 4). To confirm that the in vitro degradation of TnT was mediated primarily by exogenous μ-calpain, myofibrils were incubated with the protease in the presence of EDTA for 0, 2, 60, 1,440, or 2,880 min. Western blotting revealed that the amount of intact TnT in myofibrils incubated with μ-calpain and EDTA did not change from 0 to 2,880 min (Figure 5). These data document that the proteolysis observed during incubation was predominantly due to μ-calpain. When myofibrils and purified troponin were incubated at room temperature for 2,880 min without exogenous μ-calpain, some TnT degradation was still observed (Figures 6 and 7). However, relative to samples incubated with exogenous μ-calpain, the ratio of the intact TnT to degradation products was much greater in samples incubated without exogenous μ-calpain, suggesting that residual protease activity contributed minimally to the overall TnT degradation. Therefore, the TnT degradation observed in this experiment was considered to be primarily due to exogenous μ-calpain.

It is well established that proteolytic degradation of muscle proteins is responsible for improved tenderness after postmortem storage of meat. There is, however, still debate regarding the exact enzyme or enzymes responsible for this proteolysis. Clearly, the calpain family of proteases is responsible for much of the degradation that occurs. These highly abundant enzymes have access to myofibrils, require no ATP for activation, and have the ability to reproduce postmortem changes in myofibrils in vitro (Koohmaraie, 1988; Huff-Lonergan et al., 1996). However, other workers have reported that lysosomal proteases (Calkins et al., 1987; Johnson et al., 1990) and the cysteine protease family caspases (Kemp et al., 2006) may also contribute to postmortem tenderization; however, further evidence is needed to confirm these reports. Therefore, differences observed in this study between samples with and without added

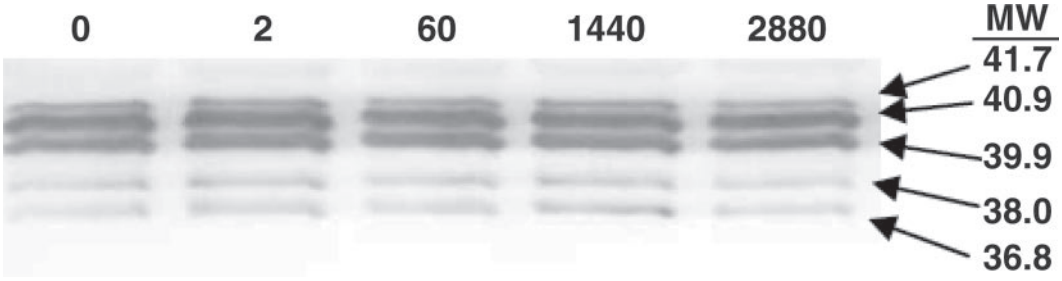

Figure 5. Western blot, prepared from a 15% resolving gel transferred to a polyvinylidene difluoride membrane, of bovine myofibrils digested with μ-calpain at a ratio of 1:800 (wt/wt; enzyme:myofibrillar protein) for 0, 2, 60, 1,440, or 2,880 min at room temperature in the presence of 100 μ*M* $CaCl_2$ and 15 m*M* β-mercaptoethanol and 20 m*M* EDTA. Blot is labeled with a monoclonal troponin T antibody (clone JLT-12, Sigma-Aldrich, St. Louis, MO). Arrows indicate molecular weights (MW) of immunoreactive bands.

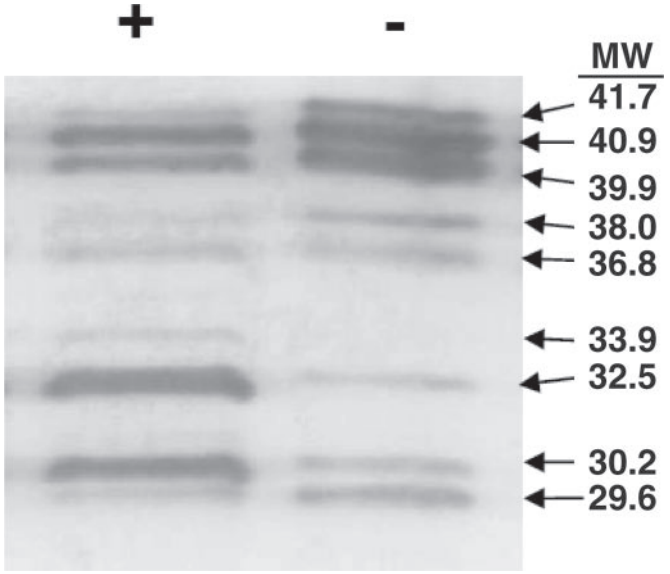

Figure 6. Western blot, prepared from a 15% resolving gel transferred to a polyvinylidene difluoride (PVDF) membrane, of bovine myofibrils incubated for 2,880 min at room temperature with μ-calpain at a ratio of 1:800 (wt/wt; enzyme:myofibrillar protein; left lane) and without exogenous μ-calpain (right lane). Both were incubated in the presence of 100 μ*M* $CaCl_2$ and 15 m*M* β-mercaptoethanol. Blot labeled with a monoclonal PVDF (troponin T) antibody (clone JLT-12, Sigma-Aldrich, St. Louis, MO). Arrows indicate molecular weights (MW) of immunoreactive bands.

μ-calpain could be the result of other endogenous protease systems that were not inhibited in this protocol. Future studies comparing the degradation of TnT by cathepsin and caspase enzymes will help determine if this is a valid hypothesis.

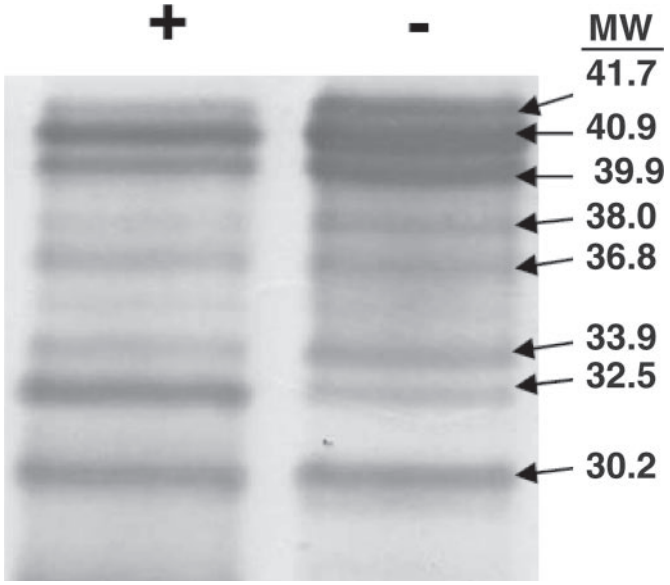

Figure 7. Western blot, prepared from a 15% resolving gel transferred to a polyvinylidene difluoride membrane, of purified troponin samples incubated for 2,880 min at room temperature with μ-calpain at a ratio of 1:800 (wt/wt; enzyme:protein; left lane) and without exogenous μ-calpain (right lane). Both were incubated in the presence of 100 μ*M* $CaCl_2$ and 15 m*M* β-mercaptoethanol. Blot labeled with a monoclonal troponin T antibody (clone JLT-12, Sigma-Aldrich, St. Louis, MO). Arrows indicate molecular weights (MW) of immunoreactive bands.

Implications

By generating myofibrils with large differences in sarcomere length and controlling the influence of proteases, we show that μ-calpain-mediated proteolysis of TnT occurs to a greater extent in samples with long sarcomeres. This is consistent with the hypothesis that proteolysis is hindered by increased overlap between the thick and thin filaments, which inhibits protease access to intrasarcomeric substrates. The mechanism by which access to cleavage sites is hindered remains unclear. These results suggest that the presence of rigor bonds does not block calpain cleavage sites on TnT. In the present study, ideal conditions were created for μ-calpain digestion in vitro. Future research simulating postmortem conditions will provide additional insight into the interaction between protein interactions and proteolysis.

LITERATURE CITED

Bendall, J. R., and C. A. Voyle. 1967. A study of the histological changes in the growing muscles of beef animals. J. Food Technol. 2:259–283.

Calkins, C. R., S. C. Seideman, and J. D. Crouse. 1987. Relationships between rate of growth, catheptic enzymes and meat palatability in young bulls. J. Anim. Sci. 64:1448–1457.

Chambers, E., and J. R. Bowers. 1993. Consumer perception of sensory qualities in muscle foods. Food Technol. 47:116–120.

Davey, C. L., and K. V. Gilbert. 1976. The temperature coefficient of beef ageing. J. Sci. Food Agric. 27:244–250.

Davey, C. L., H. Kuttel, and K. V. Gilbert. 1967. Shortening as a factor in meat aging. J. Food Technol. 2:53–56.

Ertbjerg, P., P. Henckel, A. Karlsson, L. M. Larsen, and A. J. Moller. 1999. Combined effect of epinephrine and exercise on calpain/calpastatin and cathepsin B and L activity in porcine longissimus muscle. J. Anim. Sci. 77:2428–2436.

Goll, D. E., V. F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The calpain system. Physiol. Rev. 83:731–801.

Huff-Lonergan, E., T. Mitsuhashi, D. D. Beekman, F. C. Parrish Jr., D. G. Olson, and R. M. Robson. 1996. Proteolysis of specific muscle structural proteins by micro-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. J. Anim. Sci. 74:993–1008.

Hughes, M. C., S. Geary, E. Dransfield, P. L. H. McSweeney, and E. E. O'Neill. 2001. Characterization of peptides released from rabbit skeletal muscle troponin-T by μ-calpain under conditions of low temperature and high ionic strength. Meat Sci. 59:61–69.

Huxley, H. E. 1967. Recent x-ray diffraction and electron microscope studies of striated muscle. J. Gen. Physiol. 50:71–83.

Jaime, I., J. A. Beltran, P. Cena, P. Lopez-Lorenzo, and P. Roncales. 1992. Tenderisation of lamb meat: Effect of rapid postmortem temperature drop on muscle conditioning and aging. Meat Sci. 32:357–366.

Johnson, M. H., C. R. Calkins, R. D. Huffman, D. D. Johnson, and D. D. Hargrove. 1990. Differences in cathepsin B + L and calcium-dependent protease activities among breed type and their relationship to beef tenderness. J. Anim. Sci. 68:2371–2379.

Kemp, C. M., R. G. Bardsley, and T. Parr. 2006. Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle. J. Anim. Sci. 84:2841–2846.

Koohmaraie, M. 1988. The role of endogenous proteases in meat tenderness. Pages 89–100 in Proc. 41st Recip. Meat Conf., Laramie, WY. Natl. Livest. Meat Board, Chicago, IL.

Lametsch, R., P. Roepstorff, H. S. Moller, and E. Bendixen. 2004. Identification of myofibrillar substrates for μ-calpain. Meat Sci. 68:515–521.

Locker, R. H. 1982. A new theory of tenderness in meat, based on gap filaments. Pages 92–100 in Proc. 35th Recip. Meat Conf., Blacksburg, VA. Natl. Livest. Meat Board, Chicago, IL.

Morgan, J. B., J. W. Savell, D. S. Hale, R. K. Miller, D. B. Griffin, H. R. Cross, and S. D. Shackelford. 1991. National beef tenderness survey. J. Anim. Sci. 69:3274–3283.

Muroya, S., I. Nakajima, M. Oe, and K. Chikuni. 2006. Difference in postmortem degradation pattern among troponin T isoforms expressed in bovine longissimus, diaphragm, and masseter muscles. Meat Sci. 72:245–251.

Negishi, H., E. Yamamoto, and T. Kuwata. 1996. The origin of the 30 kDa component appearing during post-mortem ageing of bovine muscle. Meat Sci. 42:289–303.

Page, S. G., and H. E. Huxley. 1963. Filament lengths in striated muscle. J. Cell Biol. 19:369–390.

Perry, S. V. 1998. Troponin T: Genetics, properties and function. J. Muscle Res. Cell Motil. 19:575–602.

Ringkob, T. P., D. R. Swartz, and M. L. Greaser. 2004. Light microscopy and image analysis of the thin filament lengths utilizing dual probes on beef, chicken, and rabbit myofibrils. J. Anim. Sci. 82:1445–1453.

Tompa, P., P. Buzder-Lantos, A. Tantos, A. Farkas, A. Szilágyi, Z. Bánóczi, F. Hudecz, and P. Friedrich. 2004. On the sequential determinants of calpain cleavage. J. Biol. Chem. 279:20775–20785.

Weaver, A. D., B. C. Bowker, and D. E. Gerrard. 2008. Sarcomere length influences postmortem proteolysis of excised bovine semitendinosus muscle. J. Anim. Sci. 86:1925–1932.

Wheeler, T. L., and M. Koohmaraie. 1994. Prerigor and postrigor changes in tenderness of ovine longissimus muscle. J. Anim. Sci. 72:1232–1238.

Wheeler, T. L., and M. Koohmaraie. 1999. The extent of proteolysis is independent of sarcomere length in lamb longissimus and psoas major. J. Anim. Sci. 77:2444–2451.